

**Figure 3. Tandem Affinity Purification (TAP) of cytoplasmic dynein complexes using His-tag and SBP-tag.** mfGFP was fused to C-terminus of dynein intermediate chain (DIC). (A) Localization of DIC-mfGFP in living HeLa cells. Scale bar, 10  $\mu$ m. (B) Isolation of cytoplasmic dynein complexes. Streptavidin (SA) column chromatography isolated DIC-mfGFP and dynein heavy chain (DHC) with some contaminating proteins in 50–75 kDa range (asterisk). Ni-NTA chromatography isolated the two proteins, but many contaminants were also seen. TAP strategy with Ni-NTA and SA (Ni-NTA + SA) isolated the two proteins with a significantly reduction of contaminants in 50–75 kDa range. doi:10.1371/journal.pone.0003822.g003

## Materials and Methods

### Construction of mfGFP and fusion proteins

The gene encoding the GFP was PCR amplified from pEGFP-N1 (Clontech) and subcloned into the pCold III vector (Takara). The DNA sequence encoding octa-histidine (8 $\times$ His), streptavidin-binding peptide (SBP), and c-Myc tag were inserted after Asp173 by several steps of PCR. The DNA sequence of the product was confirmed by DNA sequencing.

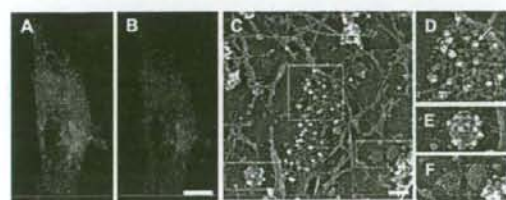
cDNAs encoding CLCA, calnexin, and DIC were obtained from human embryonic kidney (HEK) cells by RT-PCR and ligated into the pcDNA5/FRT/TO expression vector (Invitrogen). cDNA encoding rabbit RyR1 was constructed as described elsewhere [22]. The mfGFP was fused either at the C-terminus (CLCA, calnexin, and DIC), or in the middle of the coding sequence (RyR1, after Ala1397). For generation of stable transfectants, cDNA fragments for fusion proteins were ligated into pIRESHyg2 vector (Clontech). A detailed procedure of construction (with sequences of PCR primers) and full amino acid sequence of mfGFP are shown in supporting information (Supplementary Methods S1 and Supplementary Figure S1).

### Fluorescent measurements

Wild type GFP (with N-terminal hexa-histidine tag) and mfGFP were expressed in *E. coli*. The bacterial cell pellet was lysed by sonication and proteins were purified by Profinity IMAC Ni-charged resin (Bio-rad). The protein amount was measured using the Advanced protein assay reagent (Cytoskeleton Inc.) with bovine serum albumin as a standard. Fluorescence spectra of wild type GFP and mfGFP were measured in a Hitachi F-4500 fluorescence spectrophotometer in a buffer containing 150 mM NaCl and 20 mM sodium phosphate, pH 7.2.

### Cell culture

HeLa cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, and antibiotics (penicillin/streptomycin). Transfection of the expression vector was carried out using Lipofectamine LTX reagent (Invitrogen). Stable transfectants were generated by selecting colonies resistant to hygromycin after transfection of pIRESHyg2 expression vector. For isolation of protein complexes, stable and inducible HEK cell lines were generated using Flp-In T-REx system according to the manufacturer's instructions.



**Figure 4. Immunofluorescence and immuno-EM of CLCA-mfGFP expressing cells with c-Myc-specific antibody.** (A, B) Immunofluorescence microscopy. The cells were fixed and stained with c-Myc-specific antibody followed by AlexaFluor594 labeled rabbit IgG-specific secondary antibody. The AlexaFluor594 fluorescence (B) was overlapped with GFP fluorescence (A). Scale bar, 10  $\mu$ m. (C–F) Frozen-replica immuno-EM using 10 nm colloidal gold. Boxed areas in (C) are shown at higher magnification (D–F). Gold particles (white dots, marked by an arrow) were detected on the clathrin coated pits (D) and vesicles (E), but not caveolae (F) or actin cytoskeleton (C). Scale bar, 100 nm. doi:10.1371/journal.pone.0003822.g004

### Fluorescence microscopy

HeLa cells were grown on the collagen-coated coverslips. Fluorescence imaging was carried out with a confocal laser scanning microscope system (Yokogawa, CSU22) equipped with an Argon Krypton Ion Confocal Laser System (488 and 568 nm excitation). For immunofluorescent microscopy, anti-c-Myc antibody (Abcam, ab9106) and AlexaFluor594-labeled goat anti-rabbit IgG (Invitrogen, A11012) were used.

### Isolation of mGFP-tagged protein complexes

For CLCA, microsomes containing clathrin coated vesicles were prepared from HeLa cells stably expressing CLCA-mGFP according to Prasad et al [23]. Clathrin triskelions were extracted with high ionic strength solution [23] and applied onto a HiTrap streptavidin column (GE Healthcare) that had been equilibrated with buffer A (0.5 M NaCl, 20 mM MOPS, pH 7.4, 0.3 M sucrose, and 2 mM dithiothreitol). The column was washed with the buffer and the bound protein was eluted with buffer A containing 2.5 mM d-desthiobiotin. For calnexin and RyR1, microsomes prepared from the HEK cells were solubilized with buffer A containing 1% CHAPS and 0.5% soybean phosphatidylcholine, and the HiTrap streptavidin column chromatography was carried out with buffer A containing 0.5% CHAPS.

### Tandem affinity purification of dynein complex

HEK cells expressing DIC-mGFP were homogenized with buffer B (0.2 M NaCl, 50 mM Tris-HCl, pH 7.5, 10% sucrose, 5 mM MgCl<sub>2</sub>, 0.1 mM ATP, and 0.1 mM dithiothreitol) containing 0.05% Triton X-100, and the supernatant after ultracentrifugation was incubated with Profinity IMAC Ni-charged resin in buffer B containing 20 mM imidazole. After washing with the buffer, the bound protein was eluted with buffer B containing 300 mM imidazole. The eluted fraction was then applied onto a HiTrap streptavidin column that had been equilibrated with buffer C (0.2 M NaCl, 50 mM Tris-HCl, pH 7.5, 10% sucrose, 5 mM MgCl<sub>2</sub>, 0.1 mM ATP, and 0.5 mM dithiothreitol). After extensive washing, the bound protein was eluted with buffer C containing 2.5 mM d-desthiobiotin.

### Negative staining EM

Negative staining of the clathrin triskelion and RyR1 was carried out according to the standard procedures using 2% uranyl acetate. The specimens were examined in a Hitachi H-7100 electron microscope operating at 75 kV.

### Freeze-Etch EM of the Cytoplasmic Cell Surface

Rapid-freeze, deep-etch EM for the cytoplasmic surface of the plasma membrane was carried out according to previously described methods [16,24,25]. HeLa cells cultured on glass

coverslips were unroofed from the apical cell membrane and fixed. After being quenched and blocked, the cells were treated with anti-c-Myc antibody (Abcam, ab9106) followed by 10 nm gold-conjugated anti-rabbit IgG secondary antibody (GE Healthcare). Specimens were rapidly frozen to contact with the pure copper block cooled by liquid helium by using the rapid-freezing device (Polaron, USA) and the frozen cytoplasmic surface was deeply etched and rotary shadowed with platinum/carbon and carbon, using the freeze-etching device (Bal-Tec BAF060, Liechtenstein). The replica was observed by TEM (FEI Tecnai Sphera or Spirit, USA).

### Supporting Information

#### Supplementary Methods S1

Found at: doi:10.1371/journal.pone.0003822.s001 (0.03 MB DOC)

**Figure S1** Amino acid sequence of mGFP. Peptide tags inserted between Asp173 and Gly174 (boxed) are shown in color: 8×His in light blue, streptavidin-binding peptide (SBP) in yellow, c-Myc tag in green, and linkers in grey.

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**Figure S2** Design and characterization of multifunctional mCherry. (A) Schematic representation of multifunctional mCherry. Octa-histidine tag (8×His) and hemagglutinin (HA) tag were inserted in tandem after Asp174 within a loop between the β-strands (yellow) that is located on the opposite side of N- and C-termini. (B) Live-cell imaging of clathrin light chain A (CLCA)-multifunctional mCherry. Scale bar, 10 μm. (C) Detection of HA tag in immunofluorescent microscopy. The cells were fixed and stained with anti-HA antibody followed by AlexaFluor488 labeled anti-mouse IgG secondary antibody. Left, mCherry fluorescence; Right, AlexaFluor488 fluorescence. Scale bar, 10 μm. (D) Detection of HA tag in frozen-replica immuno-electron microscopy using 10 nm colloidal gold. Gold particles (white dots) were detected on the clathrin coated pits and vesicles. Scale bar, 100 nm.

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### Author Contributions

Conceived and designed the experiments: TM. Performed the experiments: TKobayashi NM TKashiyama HO NK TM. Wrote the paper: TM.

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# Mathematical morphology に基づくバイオイメージからの 構造情報の抽出と解析

## Extraction of Structural Features of Biological Images and Its Evaluation by Mathematical Morphology

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**要旨** Mathematical morphology は、画像中の物体形状を操作し計測する理論的枠組みである。本稿では、バイオイメージに対し、mathematical morphology に基づく画像処理を適用することにより、その構造情報を抽出し、定量的に計測する手法について述べる。

**キーワード**: 画像処理, mathematical morphology, 電子顕微鏡

### 1. はじめに

光学または電子顕微鏡を用いて *in vitro* 条件、あるいは組織・細胞中における生体高分子の分布や構造を捉え、それらの機能や動態を探るバイオイメージング技術は、現代のライフサイエンスにとって必須の手段である。しかし、画像データの解釈には、観察者の主観が介在し、客観的とは言い難い面もある。これは、計算機による画像処理・認識技術に未整備な部分が多いことに起因する。観察者が像を解釈する場合、経験や知識に基づいて“かたち”を読み解くプロセスを経る。しかし、従来の画像処理手法ではそれを定量的に取り扱う手立てがなく、また、互いに関連性のない様々なフィルタを寄せ集めて実行することが多く、解析に対する論理性があいまいであった。

これを踏まえ、我々は、Mathematical morphology<sup>1)</sup> (数理論形学、以降モルフォロジ) に基づく非線形的な画像解析手法を開発し、画像の定量的、客観的な評価を目指して従来とは異なる観点から新たな試みを行った<sup>2)</sup>。

モルフォロジは、集合論を基盤に、様々な画像処理を一貫した理論体系のもとで表現し実行する数理体系である。物体の“丸い”、“線維状”などのかたちの表現形式を直接取り扱うことができるため、形態に対し一定の論理的根拠を備えた定量的な解釈が可能となる。これは、輝度分布に基づく画像

処理や、周波数選択型の処理にはないユニークな特性である。また、全ての演算は、Minkowsky 和と差という単純な2項演算を基本とするため、計算機での実装が容易である。バイオイメージングの分野においてモルフォロジはまだ公知ではないが、光学顕微鏡像<sup>3)</sup>、電子顕微鏡像<sup>4)</sup> さらに DNA マイクロアレイの画像<sup>5)</sup> などに対して適用した例がある。いずれも、対象物のセグメンテーションや特徴的な構造の抽出に用いられ、効果的な処理方法が実現されている。

本稿では、様々なバイオイメージから生物学的に有意な情報を発掘・整理して観察者に提示し、新たな知見につなげるための一連のワークフローを計算機上に構築するモルフォロジカル画像処理理論について概説する。

### 2. Minkowski 集合演算とモルフォロジの基本演算

モルフォロジは、1960年代の後半、鉱物の顕微鏡画像中に分布する鉱物の幾何学的特性と物理的特性の関連を解析するための手段として G. Matheron と J. Serra より考案された。理論の整備と計算機環境の発展に伴い、1980年代になって、テキスト解析や様々な画像処理に適用されるようになった。モルフォロジは、対象画像に構造要素と呼ばれる基底関数を用いて作用させる集合論的操作により、画像中の構造を単純な“かたち”の集合として処理できることに特徴がある。これを活かし、画像中の形状に関わる特徴抽出や識別に適した手法として用いられる。モルフォロジは一般に  $N$  次元空間における集合論として展開されるが、本稿では、バイオイメージへの適用を鑑み、2次元モルフォロジ演算について説明する。

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## 2.1. 2値のモルフォロジ演算

ここでは、まず、2値のモルフォロジについて説明する。2値画像は、黒い画素(値0)の背景中に、白い画素(値1)が集まってできた物体で構成されるものとする。この物体の存在領域が関心領域である。このとき、物体は「白い画素の集合」と捉えられる。個々の白い画素は集合の要素(元)であり、画像の原点からのベクトルとして表現されるので、物体の形状をベクトルの集合として捉えなおすことができる。本稿では、集合(画像)は、アルファベットの太文字で、要素は小文字で表す。

モルフォロジの演算は以下に示すMinkowski和と差が基本になり、それらの組み合わせで演算が構成される。Minkowski和とは、2つの集合 $X, B$ があるとき、それぞれの集合の要素 $x, b$ の全ての組み合わせからできる集合のことである。集合 $X$ を $b$ だけ平行移動して得られる集合を $(X)_b$ と書くと、Minkowski和はその和集合であり、以下のよう定義できる。

$$X \oplus B = \bigcup_{b \in B} (X)_b \quad (1)$$

一方、Minkowski差は、集合 $(X)_b$ の積集合として以下のようにならされる。

$$X \ominus B = \bigcap_{b \in B} (X)_b \quad (2)$$

ここで、記号 $\oplus, \ominus$ はそれぞれ、Minkowski和、差の演算子を意味する。

また、集合 $B$ を原点を中心として180°回転させて得られる集合を、集合 $B$ の対称集合と呼ぶ。ここでは、 $B'$ と表し、以下のように定義する。

$$B' = \{-b : b \in B\} \quad (3)$$

集合 $B$ をその対称集合 $B'$ で置き換えたときのMinkowski和と差をそれぞれ、dilation, erosionと呼ぶ。これらはモルフォロジの基本演算であり、dilationは、「ずらし重ね」、erosionは「掻き取り」と訳される。dilationは次のように定義される。

$$X \oplus B' = \bigcup_{b \in B} (X)_b \quad (4)$$

一方、erosionは次のように定義される。

$$X \ominus B' = \bigcap_{b \in B} (X)_b \quad (5)$$

これらの演算の適用例を図1に示す。2値画像を表す閉集合 $X$ に、2次元の円盤状の閉集合 $B$ (構造要素)を作用させる。この場合、 $B$ は原点に対し対称であるため、dilationとerosionの結果は、それぞれ、Minkowski和と差の結果に等しくなる。 $X$ に $B$ をずらし重ねた結果、dilationは $B$ の半径分だけ外側に膨張させる。一方、erosionは $B$ の半径の大きさだけ $X$ を掻き取り、その分だけ収縮させる。また、dilationとerosionを組み合わせた演算としてopeningとclosingがある。集合 $X$ の構造要素 $B$ によるopeningを $X_B$ と表すと、以下のよう定義できる。

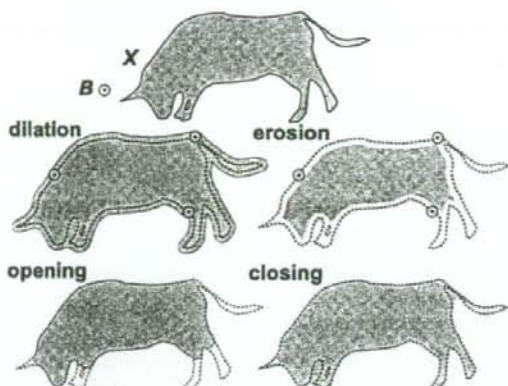


図1 原画像 $X$ の構造要素 $B$ によるdilation, erosion, openingおよびclosing演算。点線は原画像の輪郭を、灰色の領域は演算後の画像を表す。

$$X_B = (X \oplus B') \ominus B \quad (6)$$

一方、closingを $X^B$ とすると、

$$X^B = (X \ominus B') \oplus B \quad (7)$$

と定義される。これらは、dilationとerosionの演算順序が異なる。

図1にopeningとclosingの例を示す。openingは境界を滑らかにし、円盤 $B$ が入り込めないような地峡形状や尖った岬形状を除去する平滑化作用をもつ。一方closingは、 $B$ が入り込めないような間隙や穴を埋め、一方で突起領域をそのまま残す作用をもつ。

## 2.2. 多値のモルフォロジ演算

多値(濃淡)画像は、画素の座標(画像の空間領域)を $x, y$ の2つの次元で表し、画素値 $f(x, y)$ を次元 $z$ で表す3次元Euclid空間上の集合と考える。多値画像に対するモルフォロジ演算は、陰影(Umbra)という概念を用いれば、2値画像の場合の演算と平行な議論が可能となる。陰影とは、 $z \leq f(x, y)$ を満たす領域であり、これは2値集合として表現することができるため、濃淡画像が2値の集合へ変換される。

関数 $f$ の関数 $g$ によるMinkowski和は、次のようになる。

$$(f \oplus g)(x) = \max_{z \in F, z \in G} \{f(x-z) + g(z)\} \quad (8)$$

ここで、 $F, G$ はそれぞれ、関数 $f, g$ の定義域である。Minkowski差は、次のようになる。

$$(f \ominus g)(x) = \min_{z \in F, z \in G} \{f(x-z) - g(z)\} \quad (9)$$

関数 $g(x)$ の原点に対する対称関数 $g'(x)$ を定義すると以下のようになる。

$$g'(x) = g(-x) \quad (10)$$

2値のモルフォロジ演算と同様、この対称関数を用いた

Minkowski 和と差をそれぞれ dilation と erosion と呼び、多値画像に対する opening, closing もこれらを用いて定義される。

$$\text{Opening: } f_g = (f \ominus g) \oplus g \quad (11)$$

$$\text{Closing: } f^g = (f \oplus g) \ominus g \quad (12)$$

### 3. Image rotation mathematical morphology に基づく新規フィルタ

生物試料の形態は微細かつ複雑な様相を呈し、画像取得の段階で不可逆的なノイズが重畳される。それを軽減し原画像に含まれる情報を有効に利用するためには、微細構造の特性を良好に保存しつつ、背景ノイズや、画素値の変調にロバストに対応できる画像処理法が望ましい。しかし、前章で示した通常のモルフォロジ演算では構造要素の作用方向が一定のため、対象画像中に含まれる様々な向きの構造物に対し、均質な処理が困難である<sup>2)</sup>。そのまま実施すれば、解析対象の変形、構造要素の形状に依存したアーチファクトなどが生じる場合がある。

そこで我々は、モルフォロジの特性を活かしつつバイオイメージに対応できる画像処理を目指して新たなモルフォロジ演算 (Image Rotation Mathematical Morphology (IRMM) 演算)<sup>25)</sup>を開発した。これは対象画像をあらゆる方向に回転させて処理することにより、任意の方向を向いた構造の処理を可能にするものである。

いま、対象画像を  $f$ 、構造要素を  $g$  とする。  $f$  をその中心座標を回転の中心として角度  $180 \cdot i/N$  度 ( $i=0, 1, \dots, N-1$ ) ごとに回転させ、そのつど演算を行う。  $N$  は 180 度を等分した数である。結果は、各角度ごとの処理画像  $N$  枚のセットを統合して出力する。

この opening および closing 演算は以下の最大 (max)、最小 (min) 演算として定義される。

$$\text{Opening: } h_o(x, y) = \max[h_0(x, y), h_1(x, y), \dots, h_{N-1}(x, y)] \quad (13)$$

$$\text{Closing: } h_c(x, y) = \min[h^0(x, y), h^1(x, y), \dots, h^{N-1}(x, y)] \quad (14)$$

ここで、  $h_i$  は画像  $f_i$  の  $g$  による opening,  $h^i$  は closing を意味する。

#### 3.1. IRMM による平滑化フィルタ

画像処理において平滑化は重要な役割を担う。単に画像のノイズ除去の目的のみではなく、背景と対象物体の分割 (segmentation) や特徴パターン抽出の前処理として使われる。平滑化フィルタには、対象物の領域が十分に均質な濃度値で表現されるとともに、そのエッジや比較的小さな構造的な特徴を保存することが要求される。これまで、線形や非線形の様々な平滑化フィルタが提案されているが、この要求を満たすロバストな処理は困難であった。

Maragos らは、従来のフィルタでは実現できない、幾何学的な解析を行うことが大きな優位性をもつとして、モルフォ

ロジ演算を用いた平滑化フィルタを提案している<sup>6)</sup>。その具体例として opening と closing を組み合わせた、open-closing (OC) 演算、close-opening (CO) 演算があり、これらの演算をさらに線形結合した LOCO (Linear combination of OC and CO) フィルタ<sup>7)</sup>が知られる。しかし、これらのフィルタには、処理結果に前述した構造要素の形状由来のアーチファクトが生じる場合がある。それを改善するため、OC, CO 演算を式 (13, 14) で書き直し (それぞれ  $h_o, h_c, h_c, h_o$  とする)、それらを線形結合した IRMM による新たな平滑化フィルタ (SF) を定義する。

$$SF = (h_o h_c) / 2 + (h_c h_o) / 2 \quad (15)$$

図 2 はノイズ除去を目的として、原画像 (図 2a) に対し、メディアンフィルタ、LOCO フィルタおよび SF による平滑化処理の結果を比較した例である。図 2b は、メディアンフィルタでの結果である。ウィンドウサイズは、 $9 \times 9$  画素とした。ノイズの除去とともに、解析対象の輪郭がなまっている。図 2c は、LOCO フィルタでノイズを除去した結果である。やはり  $9 \times 9$  画素の正方形構造要素を用いたが、この場合も輪郭形状が変形している。さらに、構造要素の形状が重畳されアーチファクトが生じた。対象物は、6 回の回転対称性をもつため、この輪郭形状を保持するには、それぞれの突起の方向に沿った平滑化処理が必要である。SF による処理では、 $9 \times 9$  画素の正方形構造要素を用い、原画像を  $22.5$  度ずつ回転させ、そのつど平滑化処理したものを統合し出力した (図 2d)。その結果、ノイズは除去され、対象物の輪郭形状が保たれている。図 2c と比較すると、構造要素の処理方向の多方向性が結果の向上をもたらしている。

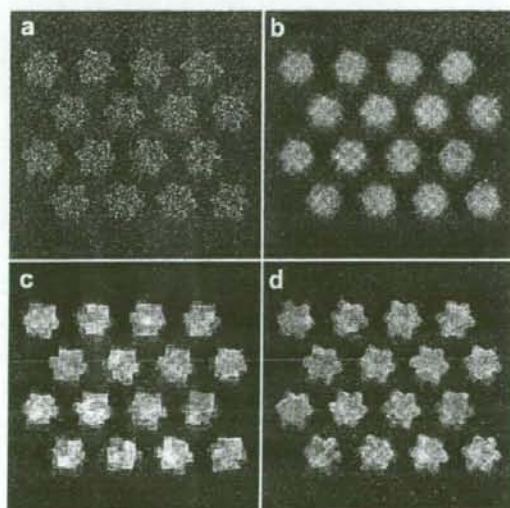


図 2 様々な平滑化処理の比較。(a) 原画像 (311×311 画素)、(b) メディアンフィルタ、(c) LOCO フィルタ、(d) IRMM による平滑化フィルタ (SF) の結果。

### 3.2. IRMMによる特徴抽出フィルタ

さらに、top-hat変換と呼ばれる演算もIRMMで定義した。

$$TH(f) = f - h_0 \quad (16)$$

top-hat変換は、原画像から、それをopeningした結果を差し引く演算である。opening ( $h_0$ ) は、一種の平滑化処理であり、構造要素に比べて小さな凸部は削られる。これは、low-pass filter であり、この出力を元の画像から引き去れば high-pass filter (opening によって削られた部分が残る) となる。構造要素のスケールを変化させるとその通過帯域が変化するという特性を活かし、画像に含まれる特定の形状の抽出に用いられる。

図3は、様々な大きさの構造要素でTHによる処理を行った例である。原画像(図3a)は、305×305画素の画像であり、画素値を高さとした複雑な地形を表現している。図3bでは、構造要素を5×5画素の正方形とすることにより、5画素以下の幅をもつ凸状の領域を抽出した。図3cでは、25画素以下、図3dでは、45画素以下の幅をもつ凸状の領域を抽出した。

構造要素をパラメータとしてTHを用いれば、このように原画像から特定の形状情報を選択的に抽出することが可能である。実際の処理では、顕微鏡などの分解能を考慮しながら抽出すべき領域の大きさを実測し、それに基づいて構造要素の大きさを決定する。

### 4. 光学顕微鏡で可視化された粒子の自動解析を可能にする画像処理

細胞内部に存在する分子に蛍光タグを融合して蛍光観察することにより、その局在や挙動を可視化できる。蛍光1分子

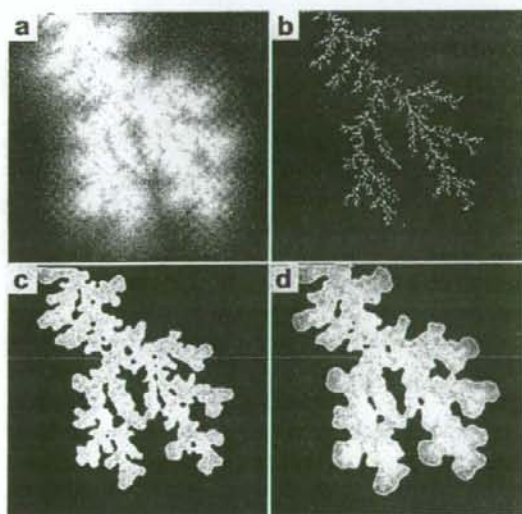


図3 IRMMによる特徴抽出フィルタ(TH)による構造抽出。(a)原画像(305×305画素)から、5画素以下(b)、25画素以下(c)、45画素以下(d)の凸状の構造を抽出した。

でも孤立した輝点として観察できるが、その分子の位置の決定には個々の輝点の像を識別して抽出する必要がある。ここでは、輝点の抽出過程を自動化するための手法を説明し、抽出した輝点を既存の解析法に適用可能なかたちに変換する手法を述べる。

実際の画像に先立ち、モデル画像に本手法を適用した例を示す。図4aは、不均一な背景画像(231×231画素)上に輝点のモデル像(半径7画素のガウス分布)を配置したものである。図中の点線は、画素値のプロファイルを計測した位置であり、以降の各処理による画素値の変化を図4eに示す。図4bは、aにガウスノイズ( $\sigma=40$ )を加えコントラストを低下させたものである。原画像とのS/N比は、12.36 [dB]であり、画素値のプロファイルを見ても、輝点と背景はほとんど区別がつかない。この画像にTHを用いた結果を図4cに示す。ただし、抽出された領域を1、他を0とする2値化を行っている。構造要素は、15×1画素の線分を用いた。top-hat変換では1階調でも差があればその領域を抽出できる。抽出したそれぞれの輝点領域の重心位置を決定し、それを頂点に半径7画素のガウス分布を発生させて輝点を復元(規格化)した(図4d)。頂点位置の誤差は、画像劣化の影響により $0.88 \pm 0.06$  (平均値±標準偏差)画素であった。従来の輝点解析手法は、輝点の点像分布関数(point spread function)をガウス関数と仮定したテンプレートマッチングを基本とする手法が多い<sup>8)</sup>。従って、この規格化および頂点位置の定量化は、既存のシステムに適用する際に重要になる。

次に、これを踏まえ培養細胞中のカベオリンに蛍光抗体を付け、光学顕微鏡で可視化した像を対象として処理した(図5)。モデル画像と同様に輝点を抽出、規格化した。図5a、bの左側は、それぞれ別の細胞の原画像(515×515画素)を、そこから輝点を抽出した結果をその右側に示す。輝点は、非常に込み合っているため、大きな幅を持つ構造要素では、近接する複数の輝点をそれぞれ独立したものとして抽出できない。そこで、構造要素は幅1画素の線状

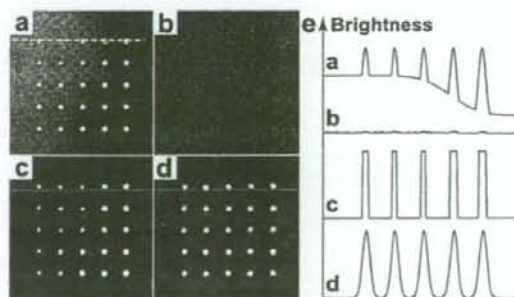


図4 輝点モデルの抽出。(a)原画像(231×231画素)。画素値のプロファイルを測定した位置を点線で示す。(b)劣化画像。(c)(b)にIRMMによる特徴抽出フィルタ(TH)を施し、輝点領域を抽出した後、2値化した。(d)輝点の復元(規格化)。(e)各処理後の画素値プロファイル。

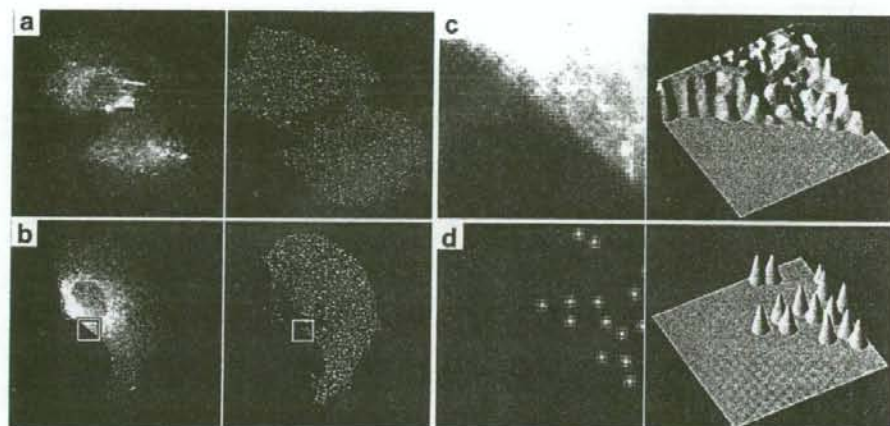


図5 光学顕微鏡画像中の輝点の抽出。(a), (b)の左側は、カペオリンを可視化した蛍光顕微鏡像(原画像, 515×515画素)。それぞれ、右は抽出された輝点の像。(c)は、(b)の左図の矩形領域の拡大。(d)は、(b)の右図の矩形領域の拡大。それぞれの右側に、画素値を高さに変換した図を表す。

とした。実際には、あらかじめ輝点のサイズを実測し、7×1画素とした。これにより、直径が7画素以下の輝点を抽出でき、また近接している場合は、それらが1画素でも離れていれば、それぞれを独立したものとして抽出できる。最終的に輝点は、半径3画素のガウス分布として規格化した。図5bの矩形領域を拡大したものが図5c, dである。図5cが原画像、図5dが抽出された輝点の画像である。それぞれの右側に輝度を高さとして示す。背景の不均一な輝度分布の中から、輝点のみが抽出されていることがわかる。既存の輝点トラッキングシステムなどへの入力に図5dを用いれば、マッチング

のミスなどが低減することが期待でき、より効果的であると考えられる。

#### 5. アクトミオシン系の解析：蛋白質複合体の電子顕微鏡画像への応用

筆者らは、精製された蛋白質の電子顕微鏡像の中から、対象蛋白質像をセグメンテーションし、あらかじめ作成された“像の見え方”のデータベース(対象蛋白質の結晶構造から構築された3Dモデルをあらゆる角度で2Dに投影した画像の辞書)とマッチングを行うという画像認識システムを構築

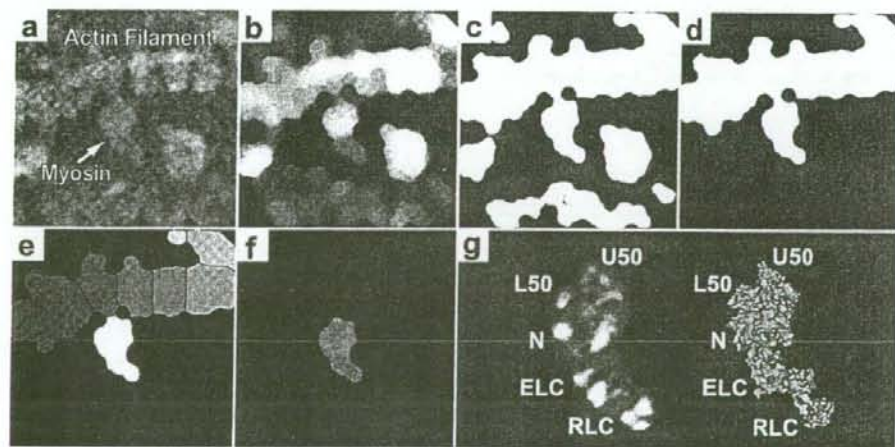


図6 蛋白質複合体への応用。(a)原画像。337×337画素。背景を含むアクチンとミオシンの複合体の電子顕微鏡像。(b)平滑化処理。(c)2値化処理。(d)複合体の領域の抽出。(e)複合体領域のセグメンテーション。(f)ミオシンの領域の抽出。(g)構造特徴(サブドメイン)を強調したミオシン像(左)と対応するモデル像(右)。主要なサブドメインを図中に示す。それぞれ、U50 (L50, upper (lower) 50-kD subdomain, Nは、NH2-terminal barrel, ELC (RLC)は essential light chain (regulatory light chain)。



した<sup>2)</sup>。ここでは、機能中の分子モーター蛋白質複合体（アクチン、ミオシン）を急速凍結ディープエッチレプリカ法で捉えた画像を対象に解析を行った例を示す。まず、原画像（図 6a, 337×337 画素）から、アクチンミオシンの複合体を抽出し、次に、複合体からのミオシン分子像のみをセグメンテーションする。このため、まず、原画像を平滑化処理し（図 6b, SF による、構造要素は直径 21 画素の円盤）、その後、自動閾値処理<sup>9)</sup>により 2 値化した（図 6c）。

その後、最大の面積をもつ領域（すなわち複合体の領域）のみを残し、あとは削除した（図 6d）。その画像に Watershed 変換<sup>10)</sup>を施すことにより、複合体の領域を複数の断片にセグメンテーションした（図 6e）。各セグメントは、それぞれ異なる濃淡値で表している（ラベル処理）。この中から、ミオシンの領域を選択し、その部分をマスクとして、原画像からミオシン像を抽出した（図 6f）。この画像を、ミオシン分子のさまざまな状態の結晶構造と比較し、最もよく合致する 3D 構造とその投影角の推定を行う。ただ、今回解析したのはアクチンの滑り運動を起こしつつある反応中間体の一瞬の構造であり、その結晶構造は得られていない。そこで、既存の結晶構造をサブドメイン単位に分割し、それらの構成部品を、電子顕微鏡像にマッチングさせ、全体として 3 次元的に合致するモデルを再構築した（未発表データ）。モデル像との照合には、ミオシンのレプリカ像そのものは用いず、分子内サブドメインで構成される構造パターン抽出した特徴画像を用いた（図 6g 左側）。構造パターンは、レプリカ像の空間分解能を考慮し、1 辺が 2 nm の正方形を構造要素として TH によって抽出した。図 6g の右側は、最もよく合致する原子モデルの像である。原子モデル像との対応により、ミオシンのサブドメイン配置の同定まで可能となった。

構造パターンを抽出した TH は、4 章で輝点を抽出したフィルタと同じである。様々な大きさや形状の異なる抽出対象物でも、構造要素を変更することにより同じ手続きで抽出が可

能であることがわかる。これは、周波数選択型の線形フィルタでは実現困難な処理であり、また、このような汎用的特徴は、ロバストなフィルタリングアルゴリズムの創出を支えている。

## 6. おわりに

本稿では、モルフォロジーの基礎演算から、バイオイメージに適すよう拡張した IRMM 演算について説明した。そして、光学顕微鏡の輝点抽出、電子顕微鏡の蛋白質複合体に適用した例を示した。数学的基盤に裏打ちされた本画像処理理論では、フィルタの設計、処理結果の解釈などに対して客観的な議論が可能となる。解析対象のかたちを定量的に取り扱い、ノイズに埋もれた情報を顕に提示する本演算法は、今後ますます必要になると思われる。

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**PART II**

Electron Microscopy of Specific  
Cellular Structure: The Cell  
Membrane

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## CHAPTER 12

# Three-Dimensional Molecular Architecture of the Plasma-Membrane-Associated Cytoskeleton as Reconstructed by Freeze-Etch Electron Tomography

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## I. Introduction

### A. General Introduction

The cytoskeleton and the plasma membrane are likely to carry out many functions *interdependently*. These structures work collaboratively, and together are largely responsible for determining the movements, shapes, and shape changes of the cell. The plasma membrane dynamics, such as endocytosis, exocytosis, membrane extension, and membrane resealing after cell wounding, could be regulated by the tension exerted on the membrane by the cytoskeleton (Sheetz and Dai, 1996; Sheetz, 2001). Signal transduction by glycosylphosphatidylinositol (GPI)-anchored proteins is mediated by the binding of stimulation-induced

clusters of GPI-anchored proteins to actin filaments (Suzuki *et al.*, 2007a,b). Tension exerted on integrin clusters in the plasma membrane by the force generated by actomyosins and by binding to the extracellular matrix strengthens the linkages between the integrin clusters and the cytoskeleton (Choquet *et al.*, 1997), possibly by way of the force-induced conformational changes of the Src family kinase substrate p130Cas at the integrin clusters (Sawada *et al.*, 2006).

The cytoskeleton has been a target of comprehensive studies by electron microscopy (EM). The "cortical" cytoskeleton, the cytoskeleton located near the plasma membrane, has also been studied quite extensively (for example, see Hartwig *et al.*, 1989; Svitkina *et al.*, 2003; Yin and Hartwig, 1988), but since many of these studies employed detergents, the structures at the exact interface between the cytoskeleton and the plasma membrane were not clear. One of the best ways to observe the membrane surface is to prepare the specimens using "freeze etching", an EM technique for sample preparation. In this technique, the biological specimen is rapidly frozen, faster than the growth of the ice microcrystals, and then, after the removal of excess ice, the ice on the membrane surface is sublimed (etching) so that the membrane surface is exposed outside the ice. The surface morphology is replicated by coating this surface with platinum, and the platinum coat is observed by EM.

This technique was greatly improved and modified for its application to investigations of the plasma membrane and the cytoskeleton near the plasma membrane by Heuser and his colleagues, as well as others, in the late 1970s and early 1980s (Chandler and Heuser, 1979; Heuser and Salpeter, 1979; Heuser *et al.*, 1979; Landis and Reese, 1981). Among them, two papers published by Heuser, Hirokawa, and their colleagues stand out in the history of the studies of both the plasma membrane and the cytoskeleton (Hirokawa and Heuser, 1981; Hirokawa *et al.*, 1982). By using intestinal epithelial tissue, and by devising various ways of identifying a variety of intracellular structures, they clearly and impressively showed images of the cytoskeleton closely associated with the microvilli and the apical plasma membrane. Actin bundles in microvilli were shown vividly. The barbed ends (fast-growing ends) were attached to the cytoplasmic surface of the tip of the villus, while the pointed ends (slowly-growing ends) at the root of the villus were linked to a structure called the "terminal web", which was further linked to the bulk cytoskeleton consisting of actin, myosin, and other intermediate filament structures. The structures linking the actin bundles as well as those linking the actin filaments and the plasma membrane were clearly shown in their electron micrographs.

Here, we list only several of the membrane-related, representative investigations using the freeze-etching technique during the last 20 years: Fujita *et al.* (2007), Hartwig *et al.* (1989), Hanson *et al.* (1997, 2008), Heuser and Anderson (1989), Heuser (2005), Italiano *et al.* (1999), Katayama *et al.* (1996), Kanaseki *et al.* (1997, 1998), Kajimura *et al.* (2000), Ohno and Takasu (1989), and Rothberg *et al.* (1992), and Nakata and Hirokawa (1992).

We have recently adopted this technique to observe the undercoat structures of the plasma membrane. In addition, we showed that the platinum replica of the rapidly-frozen, deep-etched, plasma membrane is suitable for the three-dimensional (3D) reconstruction of the cytoplasmic surface of the plasma membrane with its membrane-associated part of the cytoskeleton, using electron tomography. Hence, the objectives of this review are:

1. To show the high potential of freeze-etch EM for studying the interface between the plasma membrane and the cytoskeleton;
2. To briefly review the structure of the plasma-membrane-associated part of the cytoskeleton;
3. To briefly summarize the protocols for preparing the plasma membrane specimen with its undercoat structures, and for the rapid-freezing, deep-etching, and platinum replication of the plasma membrane specimen, with several recent improvements; and
4. To present the 3D reconstruction data and the meshwork of the actin filaments associated with the cytoplasmic surface of the plasma membrane (within 0.85 nm from the surface), and to compare the mesh size with the size of the compartments for the diffusion of plasma-membrane molecules, detected by single-molecule tracking of phospholipids and proteins.

#### **B. Introduction to Terminology: Membrane-Associated Part of the Cytoskeleton (Membrane Skeleton[MSK])**

Distinguishing the plasma membrane-associated part of the cytoskeleton from the bulk skeleton is difficult because the membrane-associated part of the cytoskeleton is continuous with the bulk cytoskeleton. In this review, we define the *membrane-associated part of the cytoskeleton as the part of the cytoskeleton that is located within several tens of nanometers from the plasma membrane, and we call this structure the "membrane skeleton (MSK)" for convenience.* The vague distance stated here is due to the large variations in the association of the cytoskeleton with the plasma membrane among different cell types.

The reasons why the term MSK, separate from the bulk cytoskeleton, is used are as follows:

1. Since the MSK-part of the cytoskeleton often functions in close cooperation with the plasma membrane, it is often more logical and easy to consider the MSK as a part of the plasma membrane, rather than as a part of the cytoskeleton, in terms of cellular functions. As mentioned in the first paragraph of this review, the proteins in the plasma membrane constantly interact with the cytoskeleton adjacent to the plasma membrane.

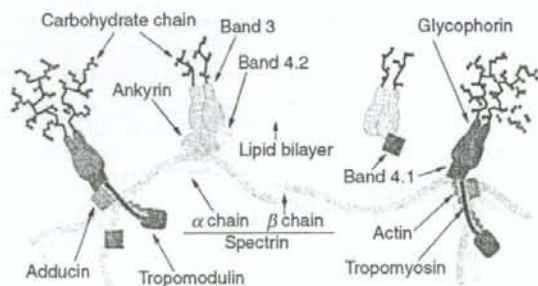
2. For the interaction with the plasma membrane, the MSK is likely to involve protein species and to have structures different from those of the bulk cytoskeleton. The proteins that are more often found in the MSK, rather than the bulk cytoskeleton, include spectrin, ankyrin, band 4.1, adducin, villin, gelsolin, supervillin, filamin, dystrophin, and utrophin.

3. Since the term membrane skeleton or MSK is often used as an adjective, such as the "MSK fence model", the following term, the membrane-associated part of the cytoskeleton, which might sound more proper, is inconvenient.

### C. Introduction to the MSK: The MSK of the Human Erythrocyte Ghost

EM investigations of the MSK structure have long been conducted using human erythrocytes. Since the human erythrocyte can easily be obtained, and since its plasma membrane can be readily isolated due to its lack of intracellular membrane compartments, the biochemical and structural analyses of the erythrocyte plasma membrane were much easier than those of other cell types. Therefore, it has been used as an important paradigm for studying the interaction between the MSK and the plasma membrane.

Due to the clarity of the results as well as the historical importance, first, we will describe the MSK structure, as revealed by EM (+AFM) as well as biochemical investigations. The MSK of the human erythrocyte even now provides a basic paradigm for studies of MSK structure and function. The schematic structure of the human erythrocyte MSK, obtained as a result of pioneering studies, is shown in Fig. 1 (Bennet, 1990; Byers and Branton, 1985). This amazing structure is completely different from the MSKs of other cell types, which will be described in the latter part of this review. Short actin filaments of approximately 40 nm in length, each consisting of 12–18 G-actin molecules, are bound by a tropomyosin



**Fig. 1** Schematic model of the MSK of the human erythrocyte. Reproduced from Morone *et al.* (2006). © 2003 The Rockefeller University Press.

molecule on its side (this molecule is considered to determine the length of these short actin filaments), and are linked to the plasma membrane on its barbed end (fast-polymerizing end) and to the tropomodulin at its pointed end (slow-polymerizing end). These filaments are densely distributed throughout the cytoplasmic surface of the plasma membrane. The short actin filaments are linked to the plasma membrane by way of protein complexes, called junctional complexes, consisting of a single-pass transmembrane protein, glycophorin C, band 4.1, and adducin, occurring most frequently every 78 nm (Byers and Branton, 1985). These junctional complexes are linked to each other sideways by flexible spectrin tetramers with lengths between 50–80 nm, which can be extended up to  $\approx 200$  nm. Schematically, this structure can be envisaged as a picket-fence structure, like a garden fence. In this analogy, the short actin filaments can be considered as the pickets, each of which is fixed to the ground by a glycophorin C-band 4.1-adducin complex, and these junctional complexes are linked by spectrin fences. The difference from the picket fences found in the agricultural farm is that the spectrin fences are not attached to the short actin pickets, but to the ground protein complex structures (junctional complexes + spectrin). The flexibility of individual spectrin molecules is considered to provide both the elasticity and resilience of erythrocytes in circulation (Evans, 1989; Mohandas and Chasis, 1993; Vertessy and Steck, 1989).

Observations of the erythrocyte's MSK without using detergent solubilization and negative staining of the erythrocyte membrane, which might modify the MSK structure greatly, have been carried out by several methods, including scanning EM (Hainfeld and Steck, 1977), thin-section EM (Tsukita *et al.*, 1980), and EM after freeze-etching and platinum replication (Nermut, 1981; Ursitti *et al.*, 1991; also see Fig. 2A in the review by Coleman *et al.* (1989), which was provided by Dr. J. Heuser of Washington University). These studies showed that the membrane skeleton is a dense, complex, three-dimensional network of filaments. Atomic force microscopy of the cytoplasmic surface of the erythrocyte plasma membrane, taking advantage of its high sensitivity to small height variations on the surface, were conducted using freeze-dried human erythrocyte ghosts (Takeuchi *et al.*, 1998). The average mesh size of the spectrin network was  $3000 \text{ nm}^2$ , which is basically consistent with the number density of junctional complexes detected by the negative-staining EM.

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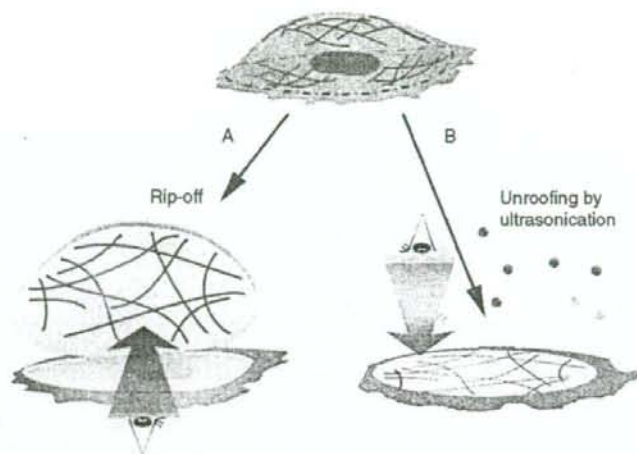
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## II. Protocol for Visualization of the Three-Dimensional Structure of the MSK of the Cytoplasmic Surface of the Plasma Membrane

### A. Methods for Exposing the Cytoplasmic Surface of the Plasma Membrane

Generally, two methods have been used for exposing the cytoplasmic surface of the plasma membrane of cultured cells, for immunolabeling and visualization of the cytoplasmic surface (Fig. 2). One is used to observe the upper plasma membrane (top surface in the cell culture on the coverslip) from inside the cell; and the





**Fig. 2** The “rip-off” method (A) and the “unroofing” method (B) for exposing and observing the cytoplasmic surface of the plasma membrane of cultured cells. Reproduced from Morone *et al.* (2006). © 2003 The Rockefeller University Press.

other is employed for the observation of the bottom cell membrane (the cell membrane facing the cover slip), again from inside the cell. The former method is often called the “rip-off” protocol, as it involves the placement and attachment of a coverslip from the top, and the subsequent removal of the top coverslip to rip the top membrane off from the rest of the cell. The latter method is referred to as “unroofing”, because the upper plasma membrane and the majority of the cytoplasmic structures and molecules are removed by very brief, weak sonication, leaving the bottom membrane attached to the coverslip on which the cell was originally cultured. Both methods can work reasonably well, but they both have their own potential problems and limitations. Further details are given in the following subsections.

Another method frequently used to visualize the actin filament meshwork and its binding proteins near the plasma membrane is to solubilize the plasma membrane using detergents. Triton X-100 solutions, containing other stabilizing reagents such as polyethylene glycol, glycerol, and sucrose, are the most popular ones. However, these detergent solutions tend to disintegrate caveolae and clathrin-coated pits. Using this detergent-solubilization method, the development of cortical actin filaments was clearly observed (Svitkina *et al.*, 1995, 2003).

a. The “rip-off” method for the observation of the cytoplasmic surface of the upper plasma membrane (Fig. 2A)

No.1 coverslips are cut into 5-mm square pieces. These small coverslips are cationized (coated) with alcian blue, a small-molecule reagent. The cationized coverslips are placed on cells cultured on the cell sheet at 4 °C for 10 min in order to allow the coverslips to attach to the plasma membrane (Fig. 3A). A buffer solution is then gently introduced into the gap between the coverslips and the cell sheet (Fig. 3B). The surface tension of the buffer forces the coverslips to float up, which could rip off the upper cell membrane from the rest of the cell, sometimes with very small amounts of the membrane skeleton or with contamination by whole cells (Fig. 3B). This buffer usually contains chemical fixatives such as 2% formaldehyde, so that the plasma membrane ripped off from the rest of the cell is immediately fixed, before its component proteins dissolve away into the rip-off buffer.

b. Unroofing by low-power ultrasonication for the observation of the cytoplasmic surface of the bottom plasma membrane (Fig. 2B)

A probe-type, low-power ultrasonic generator is used to remove the top membrane and the bulk cytoplasmic materials (Heuser, 2000). This method has often been plagued by the limited observation areas, the broken MSK meshwork

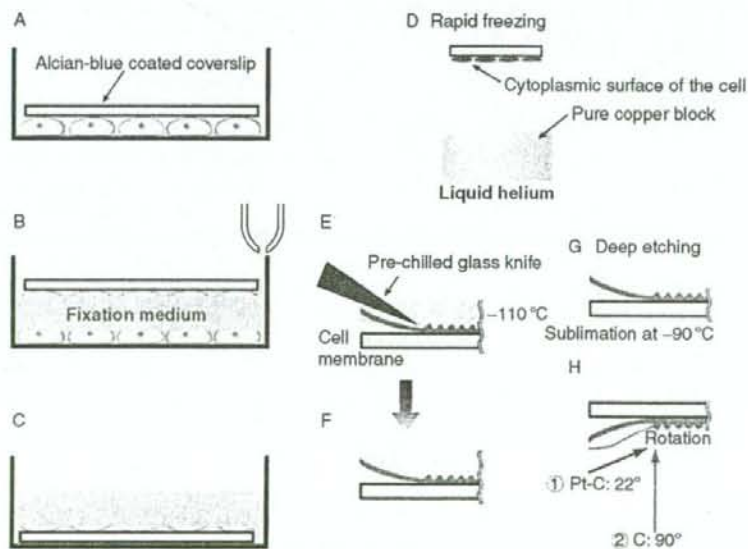


Fig. 3 Specimen preparation protocol for ripping-off the upper plasma membrane, followed by rapid-freezing, deep-etching, and platinum replication.

structures, and the loss of small membrane invaginations, including caveolae and clathrin-coated pits. Careful adjustments of the various conditions for the sonication step are required, such as the use of smaller probes (e.g., 1 mm in diameter) and lower output powers (e.g., up to several milliwatts), for the observations of the MSK, caveolae, and clathrin-coated pits spread throughout the cytoplasmic surface of the bottom plasma membrane, just like those of the upper cell membrane.

In both cases, the plasma membrane is (further) fixed by an incubation in fresh, ice-cold 1% paraformaldehyde/0.25% glutaraldehyde for 10 min (Fig. 3C).

#### B. Immunolabeling of the Proteins on the Cytoplasmic Surface of the Plasma Membrane

Immunogold labeling is an excellent way to reveal the molecular identifications in the observed structures in the electron micrograph. Immunolabeling of the isolated plasma membrane on its cytoplasmic surface can be conducted in the normal manner for fixed samples. As we intend to use platinum replication of the plasma membrane specimens, colloidal gold particles are generally recommended as probes. Since each gold particle exhibits a clear dot surrounded by a halo of platinum-carbon coating, these gold probes can be easily identified in the platinum replicated specimens. Transmembrane proteins can be labeled from the extracellular surface, and these proteins with gold probes can be retained after chemical fixation and platinum replication on the replica (Fujimoto, 1995; Fujimoto *et al.*, 1996).

#### C. Rapid-Freezing

The plasma membrane with the exposed cytoplasmic surface is next frozen quickly from the cytoplasmic surface, with its external surface still attached to the coverslip. One of the surfaces of a pure-copper block is polished with diamond paste to make a mirror surface, and the block is placed in liquid helium, with its mirror surface slightly exposed over the helium surface. Each coverslip is placed on the plunger tip of the rapid-freezing device, with the cytoplasmic surface of the membrane facing down (Fig. 3D). The plunger is slammed down onto the polished, pure-copper block (metal-contact method). This method takes advantage of the feature of pure copper, for which its thermal conductivity is maximal around the temperature of liquid-helium. Usually, within 20 microns from the frozen surface, the growth of ice crystals is sufficiently suppressed, due to the rapid freezing rate ( $10^5$  °C/s, Heuser *et al.*, 1979) so that they do not alter the cellular structures (Fig. 3D).

#### D. Deep-Etching and Platinum Replication

The frozen specimen is placed in liquid nitrogen, and then it is transferred into the freeze-etching-shadowing chamber, where the pressure can be lowered to approximately  $10^{-6}$  Pa. The excess ice covering the cytoplasmic surface of the

membrane is shaved off, with a prechilled glass or diamond knife, using a microtome placed in the chamber at  $-110^{\circ}\text{C}$  or below (Fig. 3E). Under optimal conditions, this shaving process leaves the structures (in ice) approximately 0.2 to 1 micron from the plasma membrane (Fig. 3F). The surface of the ice layer is then sublimed by slightly raising the temperature of the specimen to approximately  $-100$  to  $-70^{\circ}\text{C}$ , so that the structures hidden in the ice layer are exposed (Fig. 3G). This process is called "etching (or deep-etching as, in this protocol, the etching is more extensive than other methods)", and hence this whole specimen preparation protocol is named "freeze-etching." The etched specimen surfaces are then rotary shadowed with platinum at an angle of approximately  $20^{\circ}$  from the surface, with a thickness of 1–2 nm, and then with carbon from the top (Fig. 3H). By lowering the specimen temperature and the pressure during shadowing, the platinum grains become smaller, giving images with higher resolutions. The molecules as well as the gold probes localized on the cytoplasmic surface of the cell membrane are immobilized by the deposited platinum (Fujimoto, 1995; Fujimoto *et al.*, 1996).

#### E. Recovering the Platinum Replicas

The following is the procedure we learned from Drs. T. Baba and S. Ohno of Yamanashi University Medical School. Collodion is applied immediately after the platinum-carbon replicas are removed from the cold chamber, to fortify them. The platinum-carbon replica is removed from the glass coverslip by an incubation in 1% hydrofluoric acid in distilled water. After the replicas are successfully removed from the glass surface and mounted on the grid, the collodion coat is dissolved away in *n*-pentyl acetate. In this protocol, the sodium hypochlorite solution, which is generally used to remove the replicas from the coverslip and also to clear the membrane and the undercoat structure of the replicas, is replaced with 1% hydrofluoric acid, in order to keep the cell membrane, the undercoat structure, and the immunogold probes attached to these structures on the platinum replicas (Fujimoto, 1995; Fujimoto *et al.*, 1996; 1% hydrofluoric acid is likely to only dissolve the glass, leaving the cell membrane molecules bound to the platinum replica). An additional advantage of using 1% hydrofluoric acid is that the platinum replicas break less often, probably because the membrane components are not removed from the replicas, leaving them rather intact.

In addition, to keep as many colloidal gold particles attached to the platinum replicas as possible, all of the solutions included 0.5–1% Kodak Photo-Flo 200, a detergent used to prevent water-drop stains on the photographic film (advice from John Heuser). After the replicas are washed with distilled water, they are mounted on 100–200 mesh copper grids coated with polyvinyl formvar, and then observed at magnifications of 10,000 ~ 70,000 with a transmission electron microscope ( $\sim 80$  kV).